AD_____

Award Number: DAMD17-01-1-0250

TITLE: Anticancer Therapeutic Potential of VEGI, an Antiangiogenic Cytokine

PRINCIPAL INVESTIGATOR: Luyuan Li, Ph.D.

CONTRACTING ORGANIZATION: University of Pittsburgh
Pittsburgh, Pennsylvania 15260

REPORT DATE: October 2004

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	October 2004	Annual (10 Sep 2003 - 9 Sep 2004)

4. TITLE AND SUBTITLE
Anticancer Therapeutic Potential of VEGI, an Antiangiogenic Cytokine

5. FUNDING NUMBERS
DAMD17-01-1-0250

6. AUTHOR(S)
Luyuan Li, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Pittsburgh
Pittsburgh, Pennsylvania 15260

E-Mail: lil@upmc.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

Vascular endothelial growth inhibitor (VEGI) is an endothelial cell-specific gene and a potent inhibitor of angiogenesis and tumor growth. We are now able to produce large quantities of one of the isoforms of VEGI, VEGI-192, in E. coli. The anticancer activity of VEGI-192 was evaluated with a Lewis lung cancer murine tumor model. Systemic administration of the recombinant protein to tumor-bearing C57BL black mice by intraperitoneal injection at 20 mg/Kg, two times a week, gave rise to a marked inhibition of tumor growth. Similar efficacy was observed in the treatment of both early and late stages of the established tumors. As much as 50% inhibition of the tumor growth rate was achieved when the tumor volumes reached nearly 5% of the body weight at the time of the initiation of the treatment. Inhibition of tumor formation was also observed when the recombinant protein was given at the time of cancer cell inoculation. Immunohistochemical analysis of the tumor vasculature indicated that VEGI treatment specifically eliminated endothelial cells. Vascular smooth muscle cells in contrast were largely unaffected, and remained associated with a residual vascular structure consisting of blood vessel basement membrane. These results demonstrate the potential of recombinant VEGI-192 as a therapeutic agent for cancer treatment.

14. SUBJECT TERMS
Antiangiogenesis, preclinical, cytokine, endothelial, therapeutic

15. NUMBER OF PAGES
11

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

Report

DAMD17-01-1-0250

Introduction

We previously reported the discovery of an endothelial cell-specific gene product, vascular endothelial cell growth inhibitor (VEGI; TNFSF15), which exhibits 20%–30% sequence homology with the tumor necrosis factor superfamily (1, 2). VEGI mRNA was found in many normal adult tissues, suggesting a physiological role for this unique gene in the maintenance of the normal vasculature (3). We demonstrated that VEGI is a potent and specific inhibitor of endothelial cell growth (1-3). VEGI exhibits two distinctly different activities on endothelial cells: growth arrest of G0/G1 cells and apoptosis of proliferating cells (4). These findings suggest that VEGI may have an important role in the regulation of vascular homeostasis.

There are three differential splicing variants of VEGI (4). The VEGI gene product we initially described is a protein composed of 174 amino acids (1, 2). Hydrophobic analysis predicted VEGI-174 to be a type II transmembrane protein with residues 29-174 comprising the extracellular domain, similar to most TNF family members (5). Recombinant VEGI comprising only the putative extracellular domain exhibited an effective inhibitor of endothelial cell proliferation in culture. Full-length VEGI-174 was found, however, to have no effect on tumor growth when overexpressed in cancer cells (2), suggesting that VEGI-174 was retained by the cancer cells. On the other hand, a secretable fusion protein comprising a secretion signal peptide and the putative extracellular domain of VEGI-174 (sVEGI) was able to inhibit tumor growth when overexpressed in cancer cells (2). These findings indicate that a solubilized extracellular domain of VEGI is responsible for its biological activity. We further determined the structure of the human VEGI gene and discovered two new isoforms, VEGI-251 and VEGI-192 (3). All three isoforms exhibit an endothelial cell-specific expression pattern similar to that of the initially discovered VEGI-174. These isoforms differ in their N-terminal regions but share a 151-amino acid residue domain. VEGI-251, the most abundant isoform, is a secreted protein. Overexpression of VEGI-251 causes endothelial cell apoptosis and inhibition of tumor growth.

We evaluated the anti-cancer activity of VEGI-192 using a Lewis lung cancer murine tumor model. Systemic delivery of the protein preparation gave rise to a marked inhibition of the tumor growth. The treatment led to in specific elimination of endothelial cells but not vascular smooth muscle cells. Furthermore, we found that a prominent basement membrane "sleeve" of the existed vascular structure persisted in the tumors. These findings are consistent with the view that VEGI-192 prevents neovascularization but has no effect in triggering the destruction of existing vasculature.

Body

Inhibition of established tumors: We determined the anticancer activity of recombinant VEGI-192 with a Lewis lung cancer (LLC) murine tumor model, using immunologically intact C57BL black mice. Subcutaneously implanted LLC cells formed rapidly growing tumors. We treated the tumor-bearing animals by either topical administration

(intratumoral, IT) or systemic administration (intraperitoneal, IP). We first determined the impact of the systemically administered recombinant VEGI-192 (5 mg/Kg) on tumor formation rate. We found that IP administration of VEGI-192 at the time of the cancer cell inoculation resulted in a marked inhibition of tumor in-take rate (Figure 1A). When the tumor volumes were assessed on day 5 post inoculation, all the animals in the vehicle-treated group had developed subcutaneous tumors (5/5 or 100%), with a mean volume (+/- SD) of 35 mm^3, whereas about one-half of the VEGI-treated group exhibited measurable tumors (2/6 or 33%), and the tumor volumes were much smaller. The results indicate that systemic administration of VEGI led to retardation of tumor formation by the cancer cells.

We then determined the ability of VEGI-192 to inhibit the growth of established tumors. In one experiment, the treatment was initiated at an early time when the tumors were palpable (Figure 1B). The animals were treated on day 5, day 9, and day 12 by IP administration of VEGI-192 (5 mg/Kg). The control group was treated with vehicle. A significantly slower tumor growth rate was observed for the VEGI-treated group. In another experiment, the treatment was initiated when the tumors reached about 5% of the body weight (Figure 1C). The animals were treated two times at a dosage of 5 mg/Kg on day 11 and day 14 post tumor inoculation. We observed about 60% decrease of the tumor growth rate within one week. Comparable inhibition of the tumor growth rates were obtained using either IT or IP treatments. These data strongly suggest that systemically delivered VEGI was able to inhibit the growth of established tumors.

Specific eradication of endothelial cells in tumor: We determined the impact of systemic treatment of the LLC tumor-bearing mice with VEGI on the abundance and structure of the tumor blood vessels. Freshly frozen tumors were sectioned and subjected to immunostaining for endothelial marker CD31 (red) and smooth muscle cell antigen-a (SMA-a) (green) (Fig 2A, 2B). We then analyzed 15 fields on each slide that contained the most number of microvessels ("hot spots") by computer-assisted image analysis. The densities of the red or green pixels per field (400x magnification) were determined (Figure 2C). The results indicate a specific elimination of endothelial cells in tumor blood vessels. The density of the endothelial cells, measured as the total pixels occupied by CD31-positive cells, exhibited an 88% decrease within one week of treatment, and a further decrease within three weeks. Interestingly, the number of the smooth muscle cells remained relatively unchanged. As a result, the ratio of endothelial cells to smooth muscle cells decreased markedly in VEGI-treated tumors, changing from 1.8 to 0.4 and 1.8 to 0.15 after the animals had been treated for one or three weeks, respectively. We carried out a similar immunostaining for another endothelial cell marker, CD105, and obtained identical results (data not shown). These results indicate that the antiangiogenic activity of VEGI caused specific eradication of tumor vascular endothelial cells.

Persistence of vascular smooth muscle cells in tumors after elimination of endothelial cells: Interestingly, we found that vascular smooth muscle cells persisted for the duration of the experiment after the endothelial cells were eliminated (Figure 7). The lumens of some of the tumor vasculature appeared to be somewhat maintained. This raised the possibility that the tumor blood vessels with good smooth muscle cell support may have retained some residual function after most of the endothelial cells were no longer present.

This finding suggests that the tumor blood vessels whose endothelial cell lining has been eradicated may still perform certain functions to support circulation in the tumors. It is also plausible that the remaining vascular smooth muscle cells may provide a framework for the repair of damaged blood vessels by endothelial progenitor cells. This finding may have important implications in clinical settings where an anticancer drug is used in combination with an antiangiogenic agent.

Presence of residual vascular structures in VEGI-treated tumors: Prompted by the persistent existence of smooth muscle cells in the tumors of VEGI-treated animals, we investigated the residual vascular structure. Initially we found that, unlike the lumens of the blood vessel in the vehicle-treated tumors which were always lined by endothelial cells (Figure 3A), there were spaces in the VEGI-treated tumors that contained red blood cells but were not lined by endothelial cells (Figure 3B). We then determined whether these spaces were residual blood vessels that were depleted of endothelial cells. This is accomplished by immunostaining for collagen IV, a major component of the basement membrane of a blood vessel (McDonald). We found that the basic structure of the tumor vasculature remains nearly intact even when the endothelial cells were nearly completely eliminated by VEGI treatment (Figure 3C), as compared with the readily detectable endothelial cells that were associated with the blood vessel basement membrane in the vehicle-treated tumors of the control group (Figure 3D). These basement membrane structures were often accompanied by smooth muscle cells regardless whether the tumors were treated with VEGI-192 (Figure 3E) or vehicle (Figure 3F). The appearance of these residual vessels was not distinguishable from those seen in the untreated tumors, with the only difference that the vascular structures in the untreated tumors contain endothelial cells. These results indicate that a residual vascular structure consisting of basement membrane and smooth muscle cells exists, at least for the duration of the experiment, after endothelial cells are removed from these tumor vessels.

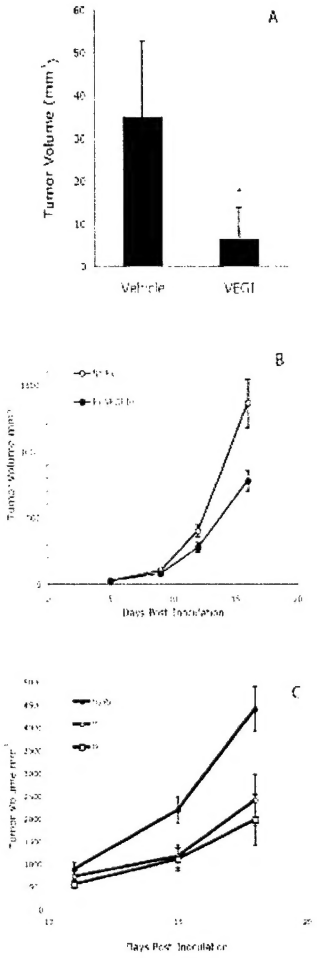

Figure 1: Inhibition of Lewis lung cancer tumor formation and growth. Panel A: Inhibition of LLC tumor formation. LLC cells ($2x10^6$ per injection per animal) were inoculated on the flank of a C57BL black mouse on Day 0. The animals were treated with recombinant VEGI-192 (20 mg/kg) immediately following cancer cell inoculation. The treatment was repeated daily until Day 4 when the tumor volumes were determined. Asterisks: T-test, $p<0.002$ (untreated, n=5; treated, n=6). Panel B: Inhibition of the growth of newly implanted LLC tumors. LLC cells ($2x10^6$ per injection) were inoculated on the flank of a C57BL black mouse on Day 0. Recombinant VEGI (20 mg/kG) was given on Days 5, 9, and 12 (arrows) by intraperitoneal (IP) injection. Tumor volumes were measured immediately prior to VEGI treatment. Asterisks: T-test, $p < 0.05$ (treated groups n = 9, untreated group n = 9). Panel C: Inhibition of the growth of established LLC tumors. LLC cells ($2x10^6$ per injection) were inoculated on the flank of a C57BL black mouse on Day 0. Recombinant VEGI (20 mg/kG) was given on Day 11 and Day 14 (arrows) by either intratumoral (IT) or intraperitoneal (IP) injection. Tumor volumes were measured on Day 15 and Day 18. Asterisks: T-test, $p < 0.05$ (treated groups n=9, untreated group n=9).

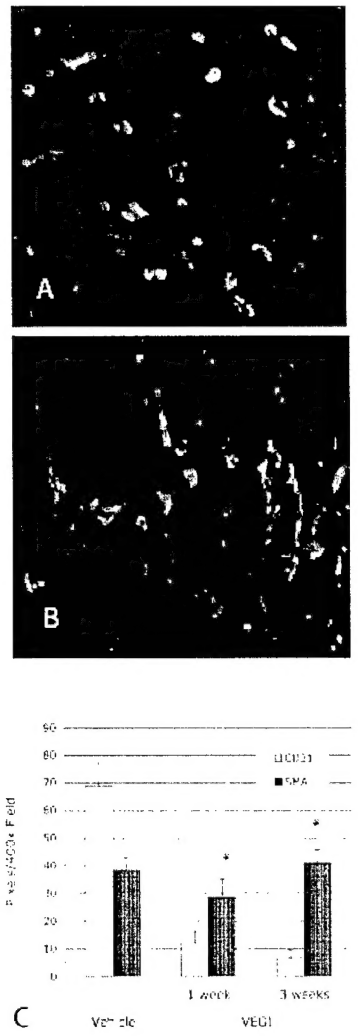

Figure 2: Specific elimination of endothelial cells by VEGI in LLC tumors. Tumors were retrieved at the end of the experiment (3 weeks) from VEGI-treated animals and vehicle-treated controls and processed as described in Methods. Sections of the tumors were subjected to fluorescent immunostaining. Endothelial cells and smooth muscle cells were identified with specific markers CD31 (red) and SMA (green), respectively. Panel A: Image of a typical tumor section from VEGI treated group; magnification, 200x. Panel B: Image of a typical tumor section from vehicle-treated group; magnification, 200x. Panel C: Quantitative analysis of red and green areas of the images of the tumors. White bars, CD31-positive endothelial cells. Black bars, SMA-positive smooth muscle cells. Asterisks, T-test, $p < 0.01$ between vehicle and VEGI treated groups for CD31 staining (5 animals per group; 15 areas/section analyzed).

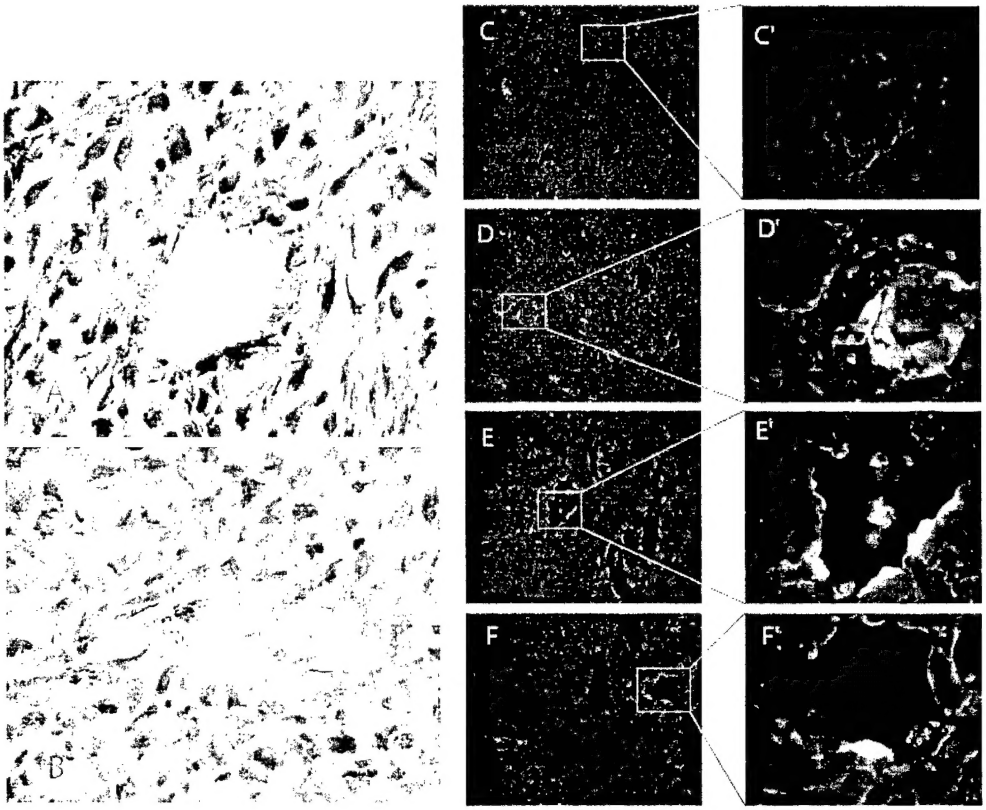

Figure 3: Presence of residual vascular structures. Sections of LLC tumors from VEGI- or vehicle-treated animals were subjected to immunostaining in order to identify endothelial cells (CD31), smooth muscle cells (SMA) and blood vessel basement membrane (collagen IV), indicating the existence of "ghost vessels" in VEGI-treated LLC tumors.. Panel A: Image of a typical vehicle-treated tumor section showing CD31-positive vessels (brown), magnification, 1000x. Panel B: Image of a typical VEGI-treated tumor section showing lumen-like spaces with red blood cells but lacked CD31-positive endothelial cells; magnification, 1000x. Panel C and C' (inset): Images of typical VEGI-treated tumor sections with CD31 (green) and collagen IV (red) double-staining; notice the lack of CD31+ endothelial cells in the lumen-like space lined by collagen IV demonstrated in C'. Panel D and D' (inset): Images of typical sections of VEGI-treated tumors with SMA (green) and collagen IV (red) staining; notice the presence of smooth muscle cells in the inner boarders of the lumen-like structures. Panel E and E' (inset): Images of typical vehicle-treated tumor sections with CD31 (green) and collagen IV (red) staining; notice the presence of endothelial cells (CD31+) in the vessel walls. Panel F and F' (inset): Images of typical vehicle-treated tumor sections with SMA (green) and collagen IV (red) staining; notice the presence of smooth muscle cells in the vessel walls. Blue staining, cell nuclei. Magnification for C, D, E, and F: 200x. Magnification for C', D', E' and F': 1000x.

Key Research Accomplishments

Our data strongly suggest that VEGI is a potentially valuable anticancer agent as it is capable of eliminating angiogenic endothelial cells in tumors when systemically administrated to LLC tumor-bearing animals. In addition, our findings support the view that residual vascular "sleeves" consisting of smooth muscle cells and blood vessel basement membrane may continue to exist in the absence of endothelial cells. Future direction of the research should be to determine whether the residual vascular structure is functional in supporting tumor circulation, and whether it provides a scaffold for the repair of the tumor vasculature damaged by antiangiogenic agents that eradicated endothelial cells.

Reportable Outcomes

US Patent filing US2002/037426

Conclusions

We demonstrated, by using the murine Lewis lung carcinoma model, that systemically delivered recombinant VEGI exhibited potent inhibitory activity on tumor formation as well as tumor growth. In one experiment, we waited until the tumor sizes reached about 5% of the body weight before the animals were treated with systemically delivered recombinant VEGI. A substantially retarded growth of the tumors were observed for the treated group during the period of about one week following the treatment, as compared to the tumor growth rate of the untreated group. This result is highly significant because similar inhibition of tumor growth was obtained when VEGI was injected directly into the base of the tumors, suggesting that the effect of VEGI was systemic. In another experiment, we treated the animals with recombinant VEGI at the time when the cancer cells were implanted. Again, recombinant VEGI was given by IP injection. Marked inhibition of tumor formation was observed with the treated group within a period of five days following VEGI injection. These findings suggest that recombinant VEGI is a potential therapeutic agent for the treatment of cancer.

References

1. Zhai, Y., Yu, J, Iruela-Arispe, L, Huang, W, Wang, Z, Hayes, A, Lu, J, Jiang, GW, Rojas, L, Lippman, ME, Ni, J, Yu, GL, Li, LY (1999) *Int. J. Cancer* **82,** 131-136.
2. Zhai, Y., Ni, J., Jiang, G., Lu, J., Xing, L., Lincoln, C., Carter, K. C., Janat, F., Kozak, D., Xu, S., Rojas, L., Aggarwal, B. B., Ruben, S., Li, L., Gentz, R. & Yu, G. (1999) *Faseb J* **13,** 181-9.
3. Chew, L. J., Pan, H., Yu, J., Tian, S., Huang, W. Q., Zhang, J. Y., Pang, S. & Li, L. Y. (2002) *FASEB Journal* **16,** 742-4.
4. Yu, J., Tian, S., Metheny-Barlow, L., Chew, L. J., Hayes, A. J., Pan, H., Yu, G. L. & Li, L. Y. (2001) *Circ Res* **89,** 1161-7.
5. Aggarwal, B. B. & Natarajan, K. (1996) *Eur Cytokine Netw* **7,** 93-124.
6. Lin, X. L., Lin, Y. Z. & Tang, J. (1994) *Methods Enzymol* **241,** 195-224.

7. Lin, X., Koelsch, G., Wu, S., Downs, D., Dashti, A. & Tang, J. (2000) *Proc Natl Acad Sci U S A* **97,** 1456-60.
8. Kim, Y. T., Downs, D., Wu, S., Dashti, A., Pan, Y., Zhai, P., Wang, X., Zhang, X. C. & Lin, X. (2002) *Eur J Biochem* **269,** 5668-77.
9. Inai, T., Mancuso, M., Hashizume, H., Baffert, F., Haskell, A., Baluk, P., Hu-Lowe, D. D., Shalinsky, D. R., Thurston, G., Yancopoulos, G. D. & McDonald, D. M. (2004) *Am J Pathol* **165,** 35-52.
10. Holash, J., Davis, S., Papadopoulos, N., Croll, S. D., Ho, L., Russell, M., Boland, P., Leidich, R., Hylton, D., Burova, E., Ioffe, E., Huang, T., Radziejewski, C., Bailey, K., Fandl, J. P., Daly, T., Wiegand, S. J., Yancopoulos, G. D. & Rudge, J. S. (2002) *Proc Natl Acad Sci U S A* **99,** 11393-8.
11. Huang, J., Frischer, J. S., Serur, A., Kadenhe, A., Yokoi, A., McCrudden, K. W., New, T., O'Toole, K., Zabski, S., Rudge, J. S., Holash, J., Yancopoulos, G. D., Yamashiro, D. J. & Kandel, J. J. (2003) *Proc Natl Acad Sci U S A* **100,** 7785-90.
12. Ferrara, N., Hillan, K. J., Gerber, H. P. & Novotny, W. (2004) *Nat Rev Drug Discov* **3,** 391-400.
13. Asahara, T., Masuda, H., Takahashi, T., Kalka, C., Pastore, C., Silver, M., Kearne, M., Magner, M. & Isner, J. M. (1999) *Circ Res* **85,** 221-8.
14. Rafii, S., Avecilla, S., Shmelkov, S., Shido, K., Tejada, R., Moore, M. A., Heissig, B. & Hattori, K. (2003) *Ann N Y Acad Sci* **996,** 49-60.
15. Rafii, S. & Lyden, D. (2003) *Nat Med* **9,** 702-12.
16. Orkin, S. H. & Zon, L. I. (2002) *Nat Immunol* **3,** 323-8.
17. Feugier, P., Jo, D. Y., Shieh, J. H., MacKenzie, K. L., Rafii, S., Crystal, R. G. & Moore, M. A. (2002) *J Hematother Stem Cell Res* **11,** 127-38.
18. Ferrara, N. (2002) *Semin Oncol* **29,** 10-4.
19. Jain, R. K. (2002) *Semin Oncol* **29,** 3-9.
20. Prehn, J. L., Mehdizadeh, S., Landers, C. J., Luo, X., Cha, S. C., Wei, P. & Targan, S. R. (2004) *Clin Immunol* **112,** 66-77.
21. Papadakis, K. A., Prehn, J. L., Landers, C., Han, Q., Luo, X., Cha, S. C., Wei, P. & Targan, S. R. (2004) *J Immunol* **172,** 7002-7.
22. Bamias, G., Martin, C., 3rd, Marini, M., Hoang, S., Mishina, M., Ross, W. G., Sachedina, M. A., Friel, C. M., Mize, J., Bickston, S. J., Pizarro, T. T., Wei, P. & Cominelli, F. (2003) *J Immunol* **171,** 4868-74.
23. Wen, L., Zhuang, L., Luo, X. & Wei, P. (2003) *J Biol Chem* **278,** 39251-8.